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PRINCIPAL INVESTIGATOR: Joanna E. Mroczkowska-Jasinska, Ph.D.

CONTRACTING ORGANIZATION: University of California at Ernest Orlando
Lawrence Berkeley National Laboratory
Berkeley, California 94720

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California at Ernest Orlando Lawrence Berkeley National Laboratory Berkeley, California 94720 E-MAIL: JEMroczkowska@lbl.gov			8. PERFORMING ORGANIZATION REPORT NUMBER	
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13. ABSTRACT (Maximum 200 Words) Over expression of the <i>erbB-2</i> (HER-2/neu) receptor occurs in up to 30% of cases of human breast cancers and correlates with aggressive disease and poor prognosis for therapy and survival. The growth factor heregulin (HRG) binds to <i>erbB-3</i> or <i>erbB-4</i> receptors, promotes dimer formation with <i>erbB-2</i> and induces autophosphorylation and activation of <i>erbB-2</i> signaling. It is generally accepted that HRG and <i>erbB-2</i> do not interact directly. Depending on its concentration HRG can either inhibit or stimulate cell proliferation in cell lines that overexpress <i>erbB-2</i> . This suggests some type of direct interaction between HRG and <i>erbB-2</i> . Solution structure of HRG and other data support the existence of a low-affinity binding site within the EGF-like domain of HRG. The goal of the proposed experiments is to define the predicted sites of interaction between HRG and the <i>erbB-2</i> receptor, through generation of HRG and <i>erbB-2</i> deletion mutants. During the second year of funding, I maintained the timeline outlined in the statement of work: a) transduced deletion mutants of <i>erbB-2</i> lacking either the putative heregulin binding site or the site important for receptor heterodimerization into Ba/F3 cells and established 8 cell lines expressing different variants of <i>erbB-2</i> and b) generated 5 different point mutations of the heregulin $\beta 1$ gene and confirmed them by sequencing, and started to work on recombinant protein induction and purification. The main result of my work was to generate 8 cell lines expressing the <i>erbB-2</i> receptor containing different deletions in the extracellular domain, and 5 different point mutations within the EGF-like domain of HRG. These mutants will be used in the experiments designed to determine the mechanism of interaction between <i>ErbB-2</i> and heregulin.				
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5. INTRODUCTION

The epidermal growth factor (EGF) receptor family comprises four transmembrane tyrosine kinases (EGFR, *erbB-2*, *erbB-3* [kinase defective], and *erbB-4*) that are involved in the genesis and progression of a variety of human carcinomas. Amplification and overexpression of the *erbB-2* (HER-2/neu) receptor tyrosine kinase receptor, occurs in up to 30% of human breast cancers and has been shown to correlate with aggressive disease and poor prognosis. Despite the involvement of *erbB-2* in tumor development, its exact biological functions have yet to be elucidated. Heregulin (HRG, NDF) is a 45 kDa growth factor that is expressed in about 30% of breast carcinomas and is associated with invasion and metastasis. HRG was initially isolated as a specific ligand for *erbB-2*, however it is generally accepted that HRG does not bind directly to *erbB-2*. HRG does bind to *erbB-3* and *erbB-4* with high affinity, which promotes heterodimerization with *erbB-2*, inducing autophosphorylation and activation of *erbB-2* signaling.

It was recently found that HRG does have an extremely low affinity for *erbB-2*. The solution structure of HRG indicates an EGF-like domain of HRG with two clusters of amino acids apparently involved in receptor binding: a conserved high-affinity binding domain of 35-40 amino acid residues located in the N-terminal portion, and a region of non-conserved amino acids comprising a low-affinity binding domain in the C-terminal portion. HRG-neutralizing antibodies prepared in Dr. Lupu's laboratory directed against the C-terminus ($\alpha 1$) and the N-terminus ($\alpha 3$) blocked HRG-induced growth effects. However, the antibodies failed to block *erbB-2* receptor phosphorylation when used separately; blockage of receptor phosphorylation was observed only when the antibodies were used in combination. These observations prompted us to hypothesize that the C-terminus constitutes a site for low-affinity binding between HRG and *erbB-2*.

The extracellular region of *erb-B* receptors has been organized into a four-domain model, in which subdomain III contributes most of the determinants involved in ligand binding and signal transduction. To determine the putative site involved in the interactions between *ErbB-2* and growth factors, a number of synthetic peptides with sequences homologous to specific *erbB-2* regions (termed the RL series) were generated in Dr. Lupu's laboratory. It was shown that the peptide RL2, which was derived from a sequence in the *erbB-2* extracellular domain, was capable of specifically blocking HRG-induction of *erbB-2* tyrosine phosphorylation. Due to this fact, it is possible that this region (in the proximity of the RL2 peptide) constitutes a critical region of the *erbB-2* receptor responsible for HRG induction of *erbB-2* heterodimerization and activation.

The goal of this project is to explore the therapeutic potential of HRG for use against breast cancers, which overexpress *erbB-2*, through the:

- a) Generation of HRG mutants that will exclusively bind to *erbB-2* and
- b) Identification of the *erbB-2* functional site (site responsible for receptor heterodimerization).

Identification of the specific site of interaction between HRG and *erbB-2* as well as identification of the *erbB-2* site responsible for HRG induction of receptor heterodimerization will enhance the necessity of developing HRG-targeted agents, including HRG antagonist and/or HRG-targeted vehicles that could improve the delivery of chemotherapeutic agents.

6. BODY

The goal of the research in this proposal is to extend the ongoing studies. The following experiments are designed to shed light on the biological and molecular mechanisms by which heregulin can induce the activation of the *ErbB-2* signaling pathway of epithelial cells that leads to the aggressive forms of tumors. The original technical objectives were as follows:

- **Task 1: To generate deletion mutants of the *erbB-2* extracellular domain in order to determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation and/or its heterodimerization with other members of the *erbB* receptor family.** These studies are being accomplished by generating a series of *erbB-2* deletion mutants and subsequent transfection of the obtained constructs in a retroviral vector into the pro-B-lymphocyte cell line Ba/F3. Following the transfection the cells will be tested for their ability to grow and proliferate in the presence or absence of the wild type HRG.
- **Task 2: To generate HRG deletion mutants (Δ -HRG) and determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling.** I will generate two sets of heregulin β mutants: one at the N-terminal domain, which contains the low-affinity binding site involved in the direct type of interaction with *erbB-2* and a second at the EGF-like domain which binds to *erbB-3*, and stimulates its dimerization with other members of the receptor tyrosine kinases family. These studies will be accomplished by creating partial deletions within critical domains and subsequent point mutations to determine which amino acids are critical for interactions with the receptor. The Ba/F3 pro-B-lymphocyte cell line transfected with the *erbB* receptors will be treated with wild type and mutant HRG in order to test if the *erbB-2* signal transduction pathway is active.

STATEMENT OF WORK:

Task 1: To establish cell lines expressing deletion mutants of the *erbB-2* extracellular domain, in order to determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation and/or its heterodimerization with other members of the *erbB* receptor family.

ACCOMPLISHED OBJECTIVES FROM INITIAL TASK 1 AND 2:

A) Transfection of the generated *erbB-2* mutants lacking the putative HRG binding site into Ba/F3 cells and isolation of specific clones (Months 10-18)

During the course of the studies, I decided to introduce generated DNA constructs into the cells by infection instead of transfection. I took this approach because of the following advantages:

- It has been proven to be more efficient.
- It does not require the long isolation of specific clones.
- It yields a very high expression level of the protein of interest.

Infection of the cells with *erbB-2* mutants: To infect the cells with the *erbB-2* containing deletions within its extracellular domain, the packaging cell line PhenixE+4 was used. The cells were transfected with DNA constructs in order to obtain the functional viruses capable of infecting Ba/F3 cells. Ba/F3 cells were co-cultured for 24 h with transfected packaging cells producing high titer virus in order to increase the efficiency of the infection. Subsequently, the Ba/F3 cells were cultured with a selective antibiotic to isolate the cells that were infected. The expression of the introduced genes was confirmed by RT-PCR (figure 1A and 1B). To check the RNA expression level of *erbB-2* deletion mutants in generated cell lines an RNase Protection Assay, which determines the quantity of expressed gene was performed (figure 2).

B) Determination of the functional site of the *erbB-2* receptor that allows HRG induction of *erbB-2* phosphorylation by phosphorylation and immunoprecipitation (Months 18-24)

To determine the functional site of the *erbB-2* receptor that allows HRG induction of receptor phosphorylation, I analyzed the difference in the phosphorylation pattern of the receptor in Ba/F3 cells, expressing various *erbB-2* mutants, treated with heregulin and these not treated. The confluent cell cultures were starved with serum over night in order to reduce the background. The next day, cells were treated with HRG and subsequently lysed with lysis buffer. Protein concentrations were measured and equal amounts of protein were separated on SDS PAGE gel. The proteins were transferred to nitrocellulose membranes and blotted with specific antibodies. This part of the task appeared to be more complicated than we originally expected. This complication was due to the fact that some of selected

Abs did not bind specifically to the proteins of interest. It was therefore necessary to test a number of new antibodies and optimize the conditions in order to detect any changes in the phosphorylation profile of the receptor. We are currently in the process of completing this task.

C) Determination, by proliferation assays, of the functional site of the erbB-2 receptor that allows HRG induction of erbB-2 tyrosine phosphorylation (Months 24-32)

Determination of the functional site of the erbB-2 receptor was performed in an interleukin-3 (IL-3) dependent Ba/F3 pro-B-lymphocyte cell line. These cells, when cultured without IL-3 can survive with heregulin when the functional erbB-2 receptor is expressed. So far I have performed proliferation assays with all cell lines infected with the different mutants of erbB-2. I was able to measure different survival ratios in the cells expressing wt erbB-2 when they were cultured with or without heregulin. None of the assays has proven sensitive enough to measure any significant difference in the cell lines expressing receptors with a deletion within the extracellular domain. I will continue to work in order to achieve optimal conditions for the proliferation assay that will allow for measuring the differences more precisely. I am currently in the process of completing this task.

Task 2A: To generate point mutations in HRG and determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling. Months 1-8: To generate HRG deletion mutants.

After extensive literature studies, I decided to generate five different constructs with point mutation that would change the amino acids involved in the heregulin interactions with the receptor into alanine. Mutations were created within the EGF-like domain of HRG using the polymerase chain reaction methodology (PCR) instead of using commercial kits for site directed mutagenesis. I took this approach because it appeared to be more efficient and gave fewer false positives.

Generation of the heregulin β 2 mutants: To generate the heregulin β 2 mutants, I initially used the full-length HRG cDNA. Using specific DNA primers, generated according to the appropriate HRG sequences for each one of the point mutations, five different mutants were generated using a two-step PCR procedure. By sequence analysis, I confirmed that all desired point mutations were successfully generated and that other unexpected mutations during PCR amplifications have not occurred.

Task 2B: To express mutated HRG recombinant protein for further study of the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling. Months 8-14: To express HRG recombinant protein with point mutations.

Expression of the mutated heregulin β 2 recombinant protein: For HRG expression I decided to use the pMALTM Protein Fusion and Purification System. I took this approach because of the following advantages:

- This system has been proven to give high-level of expression of the cloned sequences.
- It enables one-step purification of the fusion protein using MBP's affinity to maltose.

To express recombinant heregulin, I cloned mutated cDNA of HRG into the pMAL vector. The clones obtained were subsequently transfected into bacteria host. I have screened a large number of clones in order to select the ones that were able to produce recombinant protein after IPTG induction. The inducible clones will be used for the large-scale protein purification. I am currently in the process of completing this task.

TASKS REMAINING TO BE PERFORMED FROM INITIAL TASKS 1 AND 2:

Task 2: To determine if the C-terminal domain of HRG has a low-affinity binding site that is specific and unique for *erbB-2*. Months 16-24: To perform phosphorylation assays to test the HRG mutants for their ability to induce tyrosine phosphorylation of the *erbB* receptors.

TASKS REMINING TO BE PERFORMED DURING THE NEXT YEAR FROM THE INITIAL STATEMENT OF WORK

Task 1: To determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation. Months 32-36: To perform receptor binding and ligand binding assays.

Task 2: To determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling. Months 24-32: To perform anchorage-dependent and -independent proliferation assays.

7. KEY RESEARCH ACCOMPLISHMENTS

- I have successfully established eight cell lines expressing *erbB-2* receptor with different deletions localized in subdomain III of the *erbB-2* extracellular domain.
- I have successfully changed, by site-directed mutagenesis, 5 amino acids that seemed to be the most important areas of HRG for the interaction with the *erbB*-receptors. The mutations were generated using the heregulin β 2 isoform.

1. Leu179Ala
2. Val180Ala
3. Lys181Ala
4. Val225Ala
5. Met226Ala

HRG WT:

DNA SEQUENCE (994-1003 bp): CTT-GTA-AAA

PROTEIN SEQUENCE (179-181 AA) LeuValLys

HRG Leu179Ala:

DNA SEQUENCE (994-1003 bp): GCT-GTA-AAA

PROTEIN SEQUENCE (179-181 AA) AlaValLys

HRG Val180Ala:

DNA SEQUENCE (994-1003 bp): CTT-GCA-AAA

PROTEIN SEQUENCE (179-181 AA) LeuAlaLys

HRG Lys181Ala:

DNA SEQUENCE (994-1003 bp): CTT-GTA-GCA

PROTEIN SEQUENCE (179-181 AA) LeuValAla

HRG WT:

DNA SEQUENCE (1133-1138 bp): GTA-ATG

PROTEIN SEQUENCE (179-181 AA) ValMet

HRG Val225Ala:

DNA SEQUENCE (1133-1138 bp): GCA-ATG

PROTEIN SEQUENCE (179-181 AA) AlaMet

HRG Met226Ala:

DNA SEQUENCE (1133-1138 bp): GTA-GCG

PROTEIN SEQUENCE (179-181 AA) ValAla

8. REPORTABLE OUTCOMES

- Establishing cell lines expressing *erbB-2* receptor with different deletions at the putative functional site.
- Generation of point mutations of heregulin at the amino acids relevant for the interactions with erbB-receptors.

9. CONCLUSIONS

The original goal of this proposal was to generate DNA deletion constructs of the *erbB-2* and heregulin sequences for subsequent functional studies and to determine their biological significance in breast cancer.

- We have successfully infected the generated deletion mutants of the *erbB-2* into the Ba/F3 cells. In all the mutants we could observe gene expression on RNA and protein level. We have tested different antibodies specific for *erbB-2* and its phosphorylated form to choose optimal conditions for further experiments that will be performed to determine the functional site of *erbB-2*.
- We have successfully substituted the amino acids important for the HRG interactions with *erbB* receptors with alanine. All five constructs are already transformed into appropriate bacteria host strains in order to express and purify the protein. Purified protein will be used to determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling.

These experiments will enable us to further studies on define the possible direct interactions between *erbB-2* and HRG. Our future work is aimed at defining the functional sites involved in the cross-talk between these proteins. Better understanding of this process can provide new strategies to stop or slow down breast cancer progression.

10. REFERENCES

Not Applicable

11. APPENDICES

Appendix I: Figures and Figure legends

Figure 1a. RT-PCR products to confirm the expression of erbB-2 constructs containing deletions in the infected BaF3 cells. Numbers correspond to the subsequent mutants; M 100 is 100 bp DNA ladder.

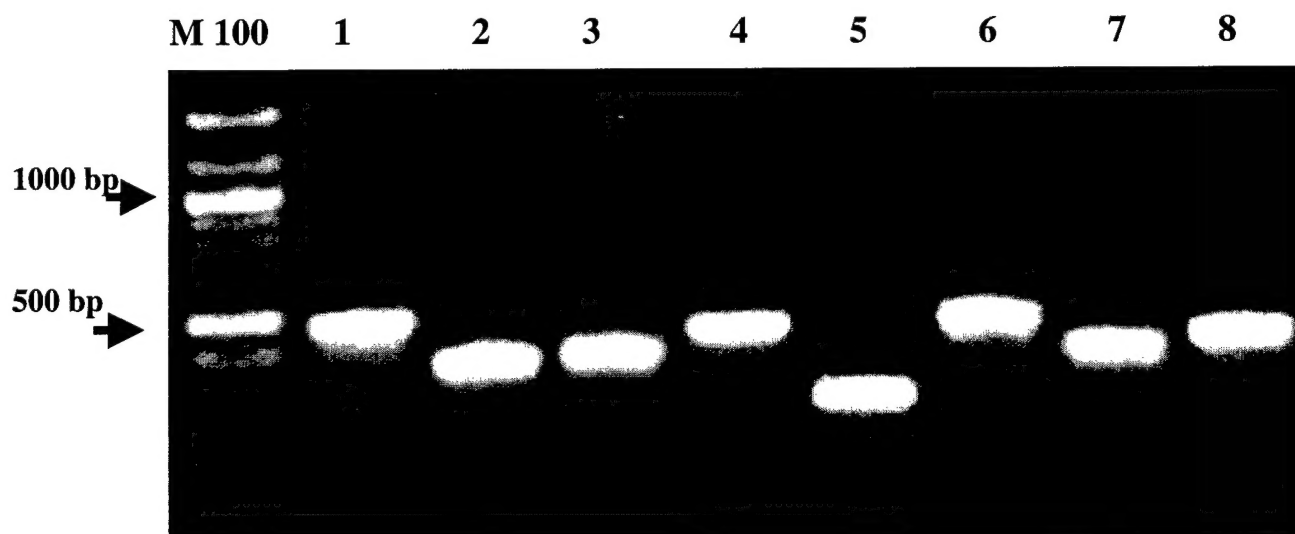


Figure 1b. PCR products of mRNA isolated from the infected cells and of the plasmids used for the infections to confirm that cells express the right erbB-2 construct. Numbers correspond to the subsequent mutants; M 100 is 100 bp DNA ladder.

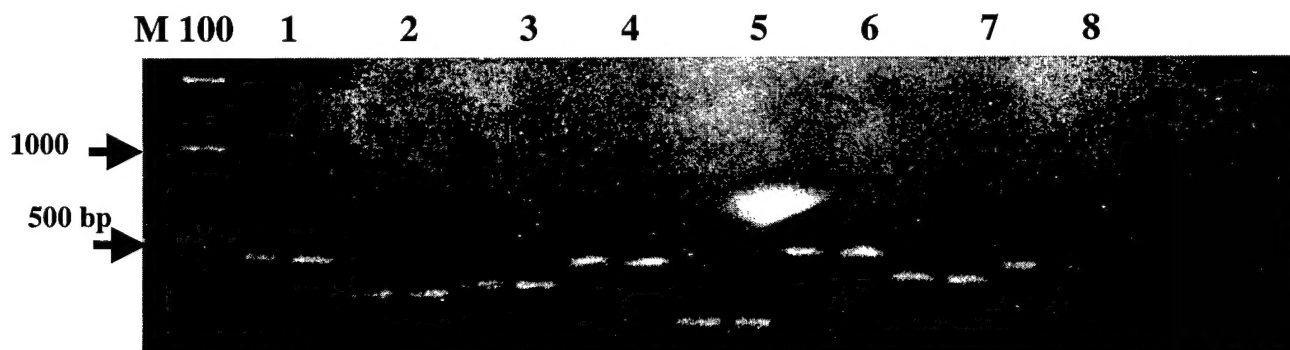


Figure 2. RNase protection assay to check the expression level of erbB-2 mutants in BaF3 cell lines. 1. Undigested riboprobe; 2-9. Subsequent mutants; 10. Cells infected with an empty vector; 11. Cells infected with wt erbB2; 12. negative control with tRNA; 13. MB-MDA 453 cell line overexpressing erbB-2 (positive control);

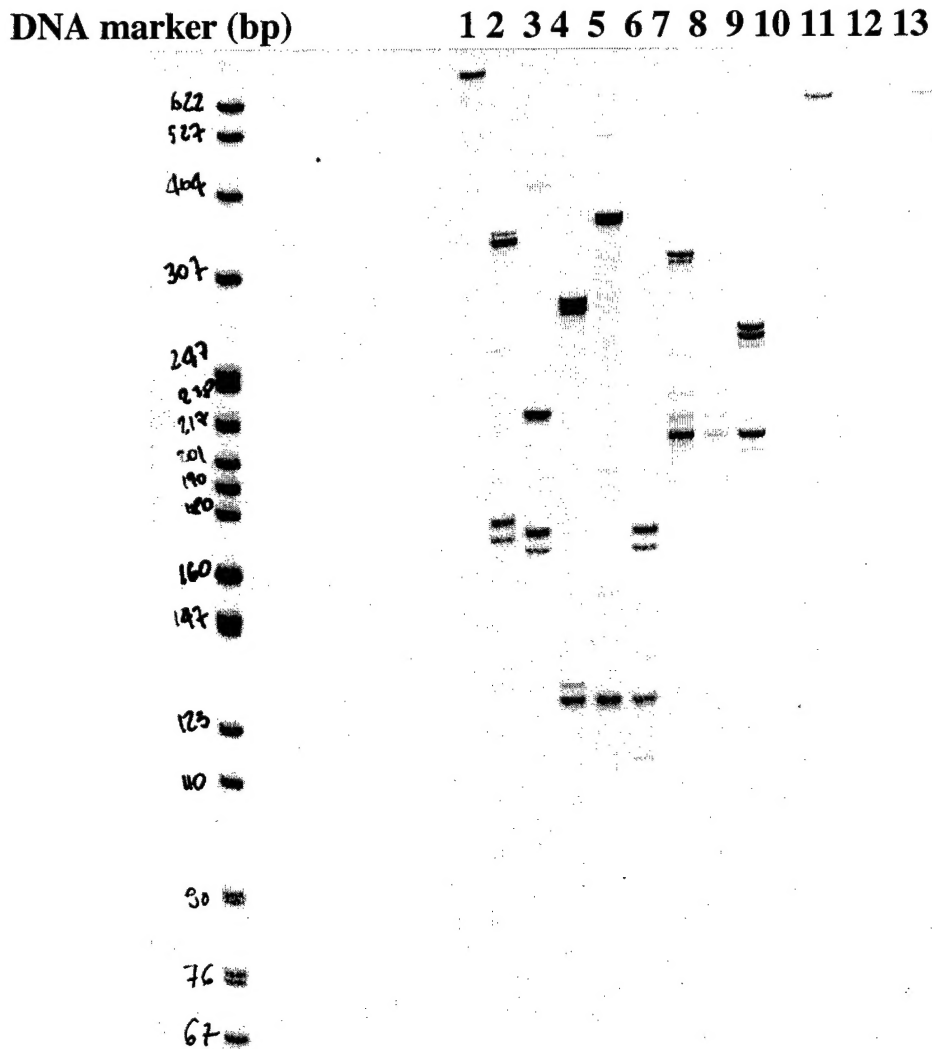


Figure 3. Western Blot to check the expression of erbB-2 mutants in BaF3 cells on protein level. Numbers 1-8 correspond to the subsequent mutants; 9. Cells infected with an empty vector; 10. Cells infected with wt erbB2; 11. MB-MDA 453 cell line overexpressing erbB-2 (positive control);

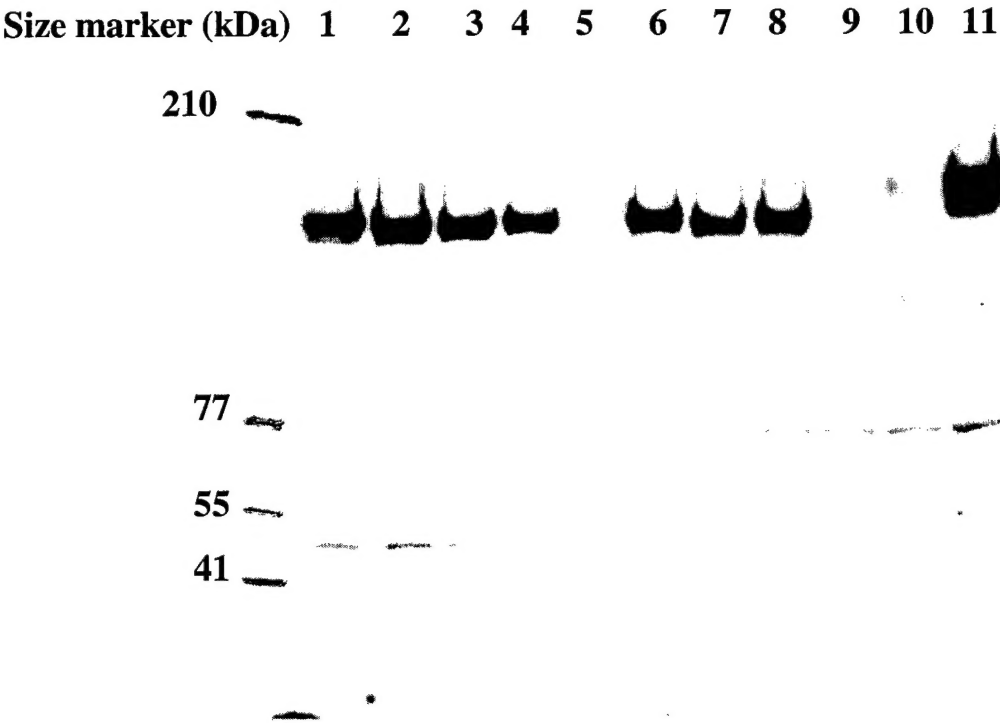


Figure 4. Immunoprecipitation with the Ab against erbB-2 protein to detect erbB-2 protein in infected BaF3 cells. Numbers 1-8 correspond to the subsequent mutants; 9. Cells infected with an empty vector; 10. Cells infected with wt erbB2; 11. MB-MDA 453 cell line overexpressing erbB-2 (positive control);

